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(54) Title: METHOD AND MATERIALS RELATING TO IMPDH AND GMP PRODUCTION

## (57) Abstract

Disclosed are purified and isolated DNA sequences encoding eukaryotic proteins possessing biological properties of inosine 5'-monophosphate dehydrogenase ("IMPDH"). Illustratively, mammalian (e.g. human) IMPDH-encoding DNA sequences are useful in transformation or transfection of host cells for the large scale recombinant production of the enzymatically active expression products and/or products (e.g. GMP) resulting from IMPDH catalyzed synthesis in cells. Vectors including IMPDH-encoding DNA sequences are useful in gene amplification procedures. Recombinant proteins and synthetic peptides provided by the invention are useful as immunological reagents and in the preparation of antibodies (including polyclonal and monoclonal antibodies) for quantitative detection of IMPDH.

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"METHOD AND MATERIALS RELATING TO IMPDH AND GMP PRODUCTION"  
BACKGROUND OF THE INVENTION

The present invention relates generally to inosine 5'-monophosphate dehydrogenase (IMPDH) and more particularly to purified and isolated DNA sequences that encode proteins possessing the biological properties of eukaryotic IMPDH, to the expression products of these DNA sequences in transformed or transfected host cells, to recombinant and synthetic proteins and peptides having amino acid sequences based on the sequence of amino acids deduced from these DNA sequences, to antibodies specific for such proteins and peptides, to analytical procedures for the detection and quantification of such peptides and proteins and nucleic acids related thereto, to the use of IMPDH-encoding DNA sequences as selectable markers and as tools for gene amplification in recombinant hosts, and to cell lines and organisms displaying enhanced production of IMPDH and/or elevated levels of products such as guanosine monophosphate (GMP), whose synthesis in cells is dependent on the activity of IMPDH.

The enzyme IMPDH (EC 1.2.1.14) catalyzes the formation of xanthine monophosphate (XMP) from inosine monophosphate (IMP). In the purine de novo synthetic pathway, IMPDH is positioned at the branch point in the synthesis of adenine and guanine nucleotides and is thus the rate-limiting enzyme in the de novo synthesis of guanine nucleotides, such as guanosine 5'-monophosphate. [Weber, Cancer Res., 43:3466-3492 (1983); Weber, et al., Adv. Enzyme Regul., 18:3-26 (1980)]. Inhibition of cellular IMPDH activity results in an abrupt cessation of DNA synthesis [Franklin, et al., Biochem. J., 113:515-524 (1969); Cohen, et al., J. Biol. Chem., 256:8713-8717 (1981); and Duan, et al., Cancer Res., 47:4047-4051 (1987)] and a cell cycl block at the G1-S interface [Cohen, et al., Cancer Res.,

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43:1587-1591 (1983)]. Because IMPDH is essential in providing the necessary precursors for DNA and RNA biosynthesis, normal tissues that exhibit increased cell proliferation generally exhibit increased IMPDH activity 5 [Jackson, et al., Nature, 256:331-333 (1975); Jackson, et al., Biochem. J., 166:1-10 (1977), Cooney, et al., Anal. Biochem., 130:339-345 (1983)]. Similarly, increased cell proliferation is accompanied by elevated enzyme activity in certain rat hepatomas with varied 10 growth rates. Weber, Cancer Res., 43:3466-3492 (1983). These hepatomas manifest IMPDH activities that are disproportionately higher than those of normal tissues, suggesting that IMPDH is associated with cell proliferation and may be linked to either malignant cell 15 transformation or tumor progression.

To investigate the role of IMPDH in growth regulation and malignancy, attempts have been made to purify the enzyme to homogeneity to allow preparation of specific antibodies thereto and to isolate IMPDH- 20 encoding DNA.

IMPDH isolated from bacterial sources has been determined to vary widely with respect to allosteric properties, size, and subunit composition. IMPDH isolated from E. coli has been purified and 25 characterized as a tetramer of identical subunits [Gilbert, et al., Biochem. J., 183:481-494 (1979); and Krishnaiah, Arch. Biochem. Biophys., 170:567-575 (1975)]. Unlike mammalian cell enzymes, the E. coli IMPDH enzyme is reported to be insensitive to the 30 inhibitory effect of mycophenolic acid [Franklin, et al., Biochem. J., 113:515-524 (1969)]. In E. coli, IMPDH has been determined to be the product of the quaB locus and the sequence of the quaB structural gene and surrounding DNA has been determined to span 1.533 kb and 35 to code for an IMPDH subunit sequence of 511 amino acids with a calculated molecular weight 54,512 [Tiedeman, et al., Nucleic Acids Research, 13:1303 (1985)].

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Miyagawa, *et al.*, Bio/Tech., 4:225 (1986), have described the cloning of the Bacillus subtilis IMPDH gene, which, upon re-introduction into a B. subtilis strain that overproduced inosine, resulted in an increased production of guanosine, accompanied by a decreased accumulation of inosine. The IMPDH gene was localized on a 6.5 kb insert and further localized to a Hind III-partially digested 2.9 kb fragment. However, the gene was not reported to have been isolated and no information was provided with respect to the DNA sequence of the gene.

While a number of workers have reported the purification or partial purification of IMPDH from a variety of eukaryotic cell sources, including ascites cells, thymus cells, mouse LS cells, and other mammalian cells, none have been successful in obtaining substantial information about the amino acid sequence of the IMPDH protein, or in establishing the utility of anti-IMPDH antibodies in the characterization of the cellular role of IMPDH.

Eukaryotic IMPDH has been obtained from one plant and several animal species, including cowpea nodule cells [Atkins, *et al.*, Arch. Biochem. Biophys., 236:807-814 (1985)], Yoshida sarcoma ascites cells [Okada, *et al.*, J. Biochem., 94:1605-1613 (1983)], rat hepatoma 3924A cells [Ikegami, *et al.*, Life Sci., 40:2277-2282 (1987) and Yamada, *et al.*, Biochem., 27:2193-2196 (1988)] and Chinese hamster cells [Collart, *et al.*, Mol. Cell. Biol., 7:3328-3331 (1987)]. The disclosures of the last-mentioned publication by the present inventors are specifically incorporated by reference herein. In all of these reports, denaturing polyacrylamide gel electrophoresis was used to assess purity and to estimate molecular weight. The reported molecular weight for all of the above mentioned enzymes was approximately 56,000. A polyclonal antibody raised

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against the purified protein was prepared for the enzyme isolated from Yoshida sarcoma ascites cells, rat hepatoma 3924A cells, and Chinese hamster cells. As described in detail, infra, only in the case of the 5 antibody prepared against the Chinese hamster enzyme was an antibody determined to be useful in examination of cellular regulation and useful in isolation of eukaryotic IMPDH-encoding DNA.

There continues to exist a need in the art for 10 information regarding IMPDH enzymes of eukaryotic origins (especially of vertebrate and more particularly of mammalian origins) such as can be provided by the isolation, sequencing, and recombinant system utilization of DNA sequences encoding the same. The 15 availability of such materials and information would make possible a vast array of novel systems and methodologies based thereon including methods and materials useful in production of products displaying IMPDH activity.

20

#### BRIEF SUMMARY OF THE INVENTION

The present invention provides novel purified and isolated DNA sequences encoding eukaryotic inosine 25 5'-monophosphate dehydrogenase (IMPDH), which have allowed for the initial determination of the primary structural conformation (i.e., amino acid sequence) of the eukaryotic protein. Specifically provided are sequences encoding human, mouse, and Chinese hamster IMPDH. Provided also are alternate DNA forms such as 30 genomic DNA and DNA manufactured by partial or total chemical synthesis from nucleotides. The association of DNA sequences of the invention with expression regulatory DNA sequences, such as promoters, enhancers 35

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and the like, allows for in vivo and in vitro transcription to form messenger RNA, which, in turn, is subject to translation to provide IMPDH protein in large quantities.

5 Among the multiple aspects of the present invention, therefore, is the provision of (a) novel eukaryotic IMPDH DNA sequences as set out in Figures 1, 2, and 3; (b) IMPDH-encoding DNA sequences, which hybridize thereto under hybridization conditions of the 10 stringency equal to or greater than the conditions described herein as used in the initial isolation of cDNAs of the invention, which also encode proteins with IMPDH biological activities; and (c) DNA sequences encoding the same, allelic variant and/or analog IMPDH 15 proteins, which incorporate, at least in part, degenerate codons. Correspondingly provided are viral or circular plasmid DNA vectors incorporating such DNA sequences and prokaryotic and eukaryotic host cells transformed or transfected with such DNA sequences and 20 vectors, as well as novel methods for the recombinant production of IMPDH proteins through cultured growth of such hosts and isolation thereof from the hosts or from their culture media.

According to another of its aspects, cell 25 lines and organisms having enhanced production of IMPDH, as well as enhanced production of guanosine monophosphate (GMP), are also provided. Preferred embodiments of such cells include the transformed or transfected host cells described initially above. Also 30 comprehended are naturally occurring or mutagenized eukaryotic cells, which are selected for increased IMPDH production (e.g., on the basis of capacity for growth in the presence of elevated levels of cytotoxic IMPDH inhibitors) and then additionally subjected to stepwise 35 incremental selection in the presence of a cytotoxic IMPDH inhibitor such as mycophenolic acid (MPA).

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ribavirin, brenidin, and tiazofurin. Illustratively, naturally occurring or mutagenized cells capable of growing in medium containing 0.1 to 0.5  $\mu$ g/mL MPA are subjected to stepwise selection at increasingly higher 5 levels of the agent.

The preparation and incorporation of IMPDH DNA sequences for use as a selectable marker to select for cells that have incorporated a selected fragment of foreign DNA into their genetic material is also embraced 10 by the present invention. In one illustration of the DNA selection systems of the invention, Chinese hamster IMPDH encoding DNA is operatively associated in a plasmid construct with appropriate expression control sequences, and e.g., a DNA sequence coding for the E. 15 coli gpt protein. This plasmid construct is then introduced into hamster cells, and cells functionally incorporating the IMPDH/gpt gene construct are selected on the basis of survival in culture media that contains MPA.

20 Novel protein products of the invention include recombinant-produced compounds having the primary structural conformation (i.e., amino acid sequence) of IMPDH protein, as well as peptide fragments thereof and synthetic peptides assembled to be 25 duplicative of amino acid sequences thereof. Proteins, protein fragments, and synthetic peptides of the invention are projected to have numerous uses including therapeutic, diagnostic and prognostic uses and also provide the basis for the preparation of monoclonal and 30 polyclonal antibodies specifically immunoreactive with IMPDH. Preferred protein fragments and synthetic peptides include those that duplicate continuous antigenic epitope sequences of the full-length protein.

35 Preferred protein products of the invention include approximately 56 kDa IMPDH peptides having the deduced sequence of 514 amino acid residues for human

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and Chinese hamster proteins set out in Figures 1 and 2. The preferred 56 kDa IMPDH polypeptide is characterized by a capacity to specifically bind IMP with a  $K_i$  equal to approximately 25  $\mu$ mol, a sensitivity 5 to inhibition by IMPDH inhibitors such as mycophenolic acid, and immunoprecipitability by rabbit anti-IMPDH antisera.

10 Antibodies specific for the novel peptide products of the invention preferably bind with high immunospecificity to IMPDH protein, fragments, and peptides, recognizing epitopes that are not common to other proteins.

15 Also provided by the present invention are novel procedures for the detection and/or quantification of the IMPDH protein, as well as the corresponding nucleic acids, e.g., DNA and mRNA, associated therewith. Antibodies of the invention may be used in known immunological procedures for quantitative detection of IMPDH protein in fluid and tissue 20 samples. DNA sequences of the invention may be suitably labeled and used for the quantitative detection of mRNA encoding IMPDH or assessment of any genetic alteration resulting in amplification or rearrangement of the IMPDH gene.

25 Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description thereof, which includes illustrative examples of the practice of the invention, reference being made to the drawing wherein:

30 Figure 1 sets forth the base sequence of a human species IMPDH cDNA and the deduced amino acid sequence of the protein product of expression of the sequence;

35 Figure 2 sets forth the base sequence of a Chinese hamster species IMPDH cDNA and the deduced amino acid sequence of the expression product thereof;

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Figures 3A and 3B set forth the base sequence for 737 bases of the 750 bases of a mouse species cDNA fragment; and,

5 Figure 4 provides a comparison of the human and Chinese hamster species IMPDH deduced amino acid sequences.

DETAILED DESCRIPTION

10 The following examples operate to illustrate practice of the invention in its numerous aspects. More particularly, Example 1 relates to the generation of cultured cell variants displaying altered levels of IMPDH activity as a result of mutagenesis and  
15 incremental selection. Example 2 relates to IMPDH purification from Chinese hamster cells and the partial purification of IMPDH from human cells. Example 3 relates to the preparation of rabbit anti-IMPDH antiserum, the isolation of IMPDH-specific IgG, and the  
20 use of this IgG in immunoblot analysis. Example 4 relates to the isolation and characterization of IMPDH cDNA from a mouse bone marrow library, a Chinese hamster library, and a human cDNA library. Example 5 relates to the protease digestion and amino terminal sequencing of  
25 the purified Chinese hamster IMPDH protein. Example 6 relates to the use of an IMPDH DNA construct as a selectable marker. Example 7 relates to the analysis of IMPDH expression in normal and in malignant cells.

30 The examples that follow are for illustrative purposes only and are not intended in any way to limit the scope of the invention.

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EXAMPLE 1

Generation of Cell Variants  
with Altered IMPDH Activity

5 To investigate the control of IMPDH enzyme in mammalian cells, cell variants were generated from the Chinese hamster V79 line of cells according to the general procedures of Huberman, et al., Proc. Nat'l. Acad. Sci. (USA), 78:3151-54 (1981).

10 To generate MPA-resistant cells,  $4 \times 10^6$  2-day-old exponentially growing V79 cells cultured in 100-mm Petri dishes were treated with the chemical mutagen/carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). After 3 hours of treatment, both the control and the MNNG-treated cells were dissociated with trypsin/ethylenediaminetetraacetic acid (EDTA) solution and seeded at  $10^5$  cells per 100-mm Petri dish. Unless otherwise noted, the cells were dissociated 6 days later and reseeded at 200 cells per 60-mm dish in 5 mL of medium for the determination of cloning efficiency and at  $2 \times 10^4$  cells per 60-mm dish in 4 mL of medium for the determination of the number of MPA-resistant cells. Two days later, MPA was added in 1 mL of medium to give a final concentration of 1  $\mu$ g/mL. Thus, an expression time of 8 days was used for the selection of MPA resistance. Cloning efficiency was determined by counting the number of Giemsa-stained colonies of six to eight dishes per point at 7-8 days after cell seeding; the number of MPA-resistant cell variants was determined by counting Giemsa-stained colonies in 40 Petri dishes per point at 18-21 days after cell seeding. The frequency of the drug-resistant colonies was calculated per  $10^5$  colony-forming cells based on the cloning efficiency and the number of cells seeded for mutant selection.

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In addition, after a 6-day expression time, a sample of control and MNNG-treated (0.5  $\mu$ g/mL) cells was incubated with MPA at 0, 0.1, and 0.3  $\mu$ g/mL. Both the control and MNNG-treated cells had a similar growth rate, yielding, after 5 days of growth, means ( $\pm$ SD) of 5.0  $\pm$  0.3, 1.4  $\pm$  0.2, and 0.2  $\pm$  0.05  $\times$  10<sup>6</sup> cells per Petri dish for MPA at 0, 0.1, and 0.3  $\mu$ g/mL, respectively. These results indicate that control and MNNG-treated cells exhibit not only a similar growth rate but also a similar susceptibility to the cytotoxic effect of MPA.

According to the present invention, the resistance level of one of these cell variants was further increased by a stepwise selection in the presence of increasing concentrations of MPA. After adaptation to the higher concentration of MPA, the cells were seeded in medium containing an increased concentration of MPA at 200 cells per 60-mm petri dish, and MPA-resistant colonies were isolated 8 days later.

Four replications of this procedure resulted in four variants, designated VM1 through VM4, which exhibited resistance to 5, 10, 25, and 50  $\mu$ g/mL MPA, respectively, whereas the parental V79 cells were resistant to only 0.1  $\mu$ g/mL MPA. The increased resistance to MPA cytotoxicity in the variant cells was associated with an increased activity of IMPDH in cell homogenates, with VM1 cells exhibiting about a six-fold increase in IMPDH activity over the parental cells, and VM2, VM3, and VM4 cells expressing about 7-, 9-, and 11-fold increases in IMPDH activity, respectively.

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EXAMPLE 2

IMPDH Purification from Chinese Hamster Cells and Human Cells

5 The generation of the IMPDH overproducing VM2 cell variant in Example 1 facilitated the isolation of a highly purified preparation of IMPDH allowing for the development of a specific anti-IMPDH antibody for subsequent cDNA cloning and immunoblot analysis.

10 IMPDH was isolated from VM2 Chinese hamster cells as follows. VM2 cells were scraped from the tissue culture plates, and washed with a phosphate buffer saline solution (PBS, pH 7.4, containing 137 mM NaCl, 2.6 mM g/L KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, and 8 mM Na<sub>2</sub>HPO<sub>4</sub>) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The cells were collected by centrifugation at 800 x g for 5 minutes and the pellet homogenized in 10 volumes of buffer A, pH 7.2, which contained 20 mM KH<sub>2</sub>PO<sub>4</sub>, 50 mM KCl, 0.5 mM dithiotreitol (DTT) and 0.1 mM PMSF. The cell homogenate was centrifuged at 180,000 x g for 40 minutes at 4°C. The supernatant was removed and applied to a hydroxylapatite column (2.5 x 30 cm) equilibrated with buffer A. The column was then washed with 10 volumes of buffer A and the protein eluted with 25 a linear gradient of 0-17.5% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (400 mL) in buffer A.

30 IMPDH activity was determined by measuring the IMP-dependent NAD reduction at 37°C by monitoring the change in absorbance at 340 nm. One unit of enzyme activity was defined as the amount of enzyme forming 1 μmole of NADH per minute at 37°C under the prescribed assay conditions. Those fractions having enzyme activity were combined and the solution adjusted to 50% saturation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. After incubation of this 35 solution at 4°C for one hour, the precipitate was collected by centrifugation at 12,000 x g for 10

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minutes. The pellet was dissolved in 50 volumes of buffer B (20 mM Tris-HCl, pH 7.0, 10% glycerol, and 0.5 mM EDTA) and the solution applied to a Blue-Sepharose™ CL-6B column (1.5 x 20 cm) equilibrated with buffer B.

5 The column was washed with 10 volumes of buffer B followed by 2 volumes of buffer B containing 10 mM NAD and 1 mM IMP. The enzyme was eluted with a linear gradient of 0-1M KCl in 200 mL of buffer B. Fractions with a high specific activity were combined,

10 concentrated by ultrafiltration, and dialyzed against buffer B. The dialyzed material was applied to a DEAE Sepharose™ column (1 x 5 cm) equilibrated with buffer B. The column was washed with buffer B and the enzyme eluted with a linear KCl gradient (0-0.5 M) in 50 mL of

15 buffer B. Protein concentration was determined by the BCA assay method (Pierce Chemical Co., Rockford, IL) with bovine serum albumin used as the standard. The enzyme was purified from a microsomal supernatant to a final specific activity of 1080 mU/mg of protein in a

20 23% yield.

Polyacrylamide gel electrophoresis was carried out in 7.5% gels as described in Laemmli, Nature, 227:680-685 (1970). Upon sodium dodecyl sulfate (SDS) gel electrophoresis, the purified protein migrated as a

25 single species with an apparent molecular weight of 56,000. Two proteins of identical molecular weight were detected by two-dimensional (2D) gel electrophoresis. One of these proteins, constituting less than 10% of the total amount of the protein, is presumably a charge-modified form of the major species. No other proteins were detected when up to one microgram of the purified protein was analyzed by electrophoresis.

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Kinetic studies were carried out by varying the substrate conditions. In all cases, linear reciprocal plots were defined by simple regression analysis of the data. The kinetic characteristics of

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the purified Chinese hamster protein were indistinguishable from those reported for the partially purified enzyme from V79 cells. The  $K_m$  values of the Chinese hamster enzyme for IMP and NAD were calculated 5 to be 21 and 29  $\mu$ M, respectively. Moreover, the Chinese hamster enzyme retained a high sensitivity to MPA with a  $K_i$  in the nanomolar range.

IMPDH was partially purified from human HL-60 cells obtained from R.C. Gallo, National Cancer 10 Institute, Bethesda, MD, and were processed as described above with respect to Chinese hamster cells. Comparison of purified Chinese hamster protein with the partially purified human enzyme by 2D-gel electrophoresis indicates these proteins are of a similar molecular 15 weight but that the human enzyme is slightly more acidic than the Chinese hamster enzyme. This observation was later confirmed by a comparison of the deduced amino acid sequences from the human and Chinese hamster cDNA clones (discussed infra), which indicated the human 20 enzyme has one fewer positively charged and one additional negatively charged amino acid.

### EXAMPLE 3

25 Preparation of Rabbit Anti-IMPDH Antiserum, Isolation of IMPDH Specific IgG, and and Development of Assay System

An anti-IMPDH antiserum was prepared in rabbits by multiple-site injections of an emulsion of the purified Chinese hamster enzyme, prepared according 30 to Example 2, and Freund's Complete Adjuvant [Vaitukaitis, Methods Enzymol., 73:46-52 (1981)]. IMPDH specific IgG was isolated from the immune serum by Protein A Sepharose™ (Pharmacia Inc., Piscataway, NJ) chromatography. By immunoblot analysis, this antibody 35 was shown to react with IMPDH isolated from Chinese hamster, rat and human cells.

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Assay systems were devised for determination of cellular IMPDH based on use of the rabbit antibody. According to these systems, target cells were washed in PBS and the cell pellet resuspended in 1-5 mL of 20 mM Tris-HCl, pH 6.8, 200 mM KCl, 1 mM DTT (buffer A). The cells were then centrifuged at 12,000 x g for 8 seconds and the pellet resuspended in 1.2 mL of buffer A. After three freeze/thaw cycles to disrupt the cells, the suspension was centrifuged at 12,000 x g for 10 minutes. The supernatant was removed and 3M NaAc, pH 5.2, was added to give a final concentration of 180 mM. After incubation on ice for 30 minutes, the protein precipitate was recovered by centrifugation for 10 min. in a microfuge. The IMPDH-enriched pellet was resuspended in 0.3 mL of 20 mM Tris-HCl, pH 8.3, 50 mM NaCl, 1 mM DTT. After removal of any insoluble material by centrifugation, aliquots were removed for the determination of enzyme activity and protein content and the remainder of the sample was stored at -20°C.

Western blot analyses [Towbin, et al., Proc. Nat'l. Acad. Sci. USA, 76:4350-4354 (1979)] were carried out with the IMPDH-specific IgG prepared from the anti-IMPDH antiserum by Protein A affinity chromatography. The detection level is approximately  $1 \times 10^{-6}$  units of IMPDH, where one unit of enzyme is defined as the amount forming 1  $\mu$ mol of product per minute at 37°C under standard assay conditions. The sample is diluted with 5x gel sample buffer (6% SDS; 200 mM DTT; 300 mM Tris-HCl, pH 6.8; 0.25% bromophenol blue; 30% glycerol) and electrophoresed at 50 volts for 12-18 hours. The proteins are transferred to nitrocellulose (Scheicher and Schuell, BA83, 0.2  $\mu$ m) in 50 mM Tris-HCl, 40 mM glycine, pH 8.3, 15% methanol by application of 100 mamps (20 V) for 8-12 hours. The nitrocellulose blot is incubated in TS (25 mM Tris-HCl, pH 7.5; 150 mM NaCl) with 5% nonfat dry milk for 30 minutes and then with the

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anti-IMPDH antibody overnight. Immune complexes were visualized by incubation with goat anti-rabbit IgG followed by incubation with rabbit IgG conjugated with horseradish peroxidase and 4-chloronaphthol (3 mg/mL in 20% methanol with 0.01% H<sub>2</sub>O<sub>2</sub>), used as the substrate. [Kittler, et al., Anal. Biochem., 137:210-216 (1984)].

EXAMPLE 4

10 Isolation and Characterization of  
IMPDH cDNA from Mouse Bone Marrow,  
Chinese Hamster, and Human cDNA Libraries

15 The rabbit antiserum prepared according to Example 3 from the purified Chinese hamster protein was used to screen a λgt11 cDNA expression library derived from mouse bone marrow [Clontech Laboratories, Inc. (Palo Alto, CA)], by means of the screening procedure outlined by Huynh, et al., DNA Cloning (Glover, D.M., ed.) 1:73-75 (1985) IRL Press Limited, Washington, D.C. The nitrocellulose filters containing the absorbed phage proteins were incubated in TS (25 mM Tris-HCl, pH 7.5; 150 mM NaCl) with 5% nonfat dry milk for 30 minutes and then with the anti-IMPDH antibody overnight. Immune complexes were visualized as described in Example 3, supra.

25 A phage with a 750 bp cDNA insert was isolated from this library and the insert subsequently subcloned into a pUC8 vector designated pUC8/IMPDH5. Confirmation of this cDNA probe as having IMPDH coding sequences was obtained by translational arrest as described in Collart, et al., Mol. Cell. Biol., 7:3328-3331 (1987). This technique indicates the extent to which the hybridization of a cDNA probe with putatively homologous mRNA can specifically reduce the yield of the translation product. Poly(A)<sup>+</sup> RNA isolated from the VM2 cells was used as a source for IMPDH mRNA. The pUC8/IMPDH5 probe effectively blocks the translation of an

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immunoprecipitable IMPDH product in a dose-dependent manner, thus validating the identity of the clone as a cDNA probe for IMPDH.

A Chinese hamster cDNA library (a generous 5 gift from Victor Ling, University of Toronto, Toronto, Canada) was prepared from a CHO cell line E29Prot (Elliott, *et al.*, *Mol. Cell. Biol.*, 5:236-241 (1985); library construction was with the pCD vector system in 10 *E. coli* x1776 [Okayama, *et al.*, *Mol. Cell. Biol.*, 2:161-170 (1982); Okayama, *et al.*, *Mol. Cell. Biol.*, 3:280-289 (1983)]. A human peripheral blood leukocyte cDNA library was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Both libraries were screened with the mouse IMPDH cDNA probe by using the procedure outlined 15 by Maniatis, *et al.*, "Molecular Cloning", Ch. 10, pages 315-317 and 320-321; Fritsch, F.T., and Sambrook, J., eds.; (Cold Spring Harbor, NY 1982).

The nitrocellulose membranes containing the recombinant DNA were prehybridized for 2 hours at 65°C 20 in a phosphate buffer, pH 7.2, containing 0.5 M Na<sub>2</sub>PO<sub>4</sub>, 1 M NaCl, 1 mM EDTA, 0.5% SDS and 100 µg/mL denatured sonicated salmon sperm DNA. The prehybridization solution was replaced with hybridization solution (prehybridization solution minus the DNA) containing 1 x 25 10<sup>6</sup> cpm/mL of <sup>32</sup>P-labeled mouse probe, prepared as described by Feinberg, A.P., *et al.*, *Anal. Biochem.*, 132:6-13 (1983), and the membranes incubated at 65°C for 36 hours. The membranes were washed 3 times for 30 min. in 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 1 mM EDTA, 180 mM NaCl at 30 50°C. The membranes were dried, sealed in plastic wrap and exposed to x-ray film (Fuji RX) with an intensifying screen (Dupont Cronex "Lightning Plus") at -70°C.

Positive plaques were purified and the phage 35 DNA isolated. Insets of recombinant IMPDH clones were excised from the positive clones with Eco RI restriction enzyme and isolated by an electro-elution technique as

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described by Zassenhaus, et al., Anal. Biochem., 125:125-130 (1982). Insert homogeneity was verified by gel electrophoresis and the concentration determined by measuring the absorbance at 260 nm.

5 The cloned mouse, human and Chinese hamster IMPDH DNAs were inserted into an M13 vector [Messing, J., Methods Enzymol., 101:20-78 (1983)] and were sequenced according to the dideoxy method [Sanger, F., et al., Proc. Nat'l. Acad. Sci. USA, 74:5463-5467 10 (1977)]. Each nucleotide sequence was read an average of four times and a minimum of once in each direction. Sequence data were compiled and analyzed by the use of the DNASTAR (Madison, WI) system digitizer and software and the accompanying protocols:

15 As illustrated in Figure 1, the largest human cDNA clone, designated HIMP, has 1642 base pairs and contains an open reading frame corresponding to a protein of 514 amino acids with a calculated molecular weight of 56,282. A consensus poly(A) addition site 20 (AATAAA) is located approximately 30 nucleotides from the termination codon at nucleotides 1584-1586. The sequence preceding the ATG methionine initiation codon at position 48 is consistent with the eukaryotic initiation site consensus sequence described by Kozak, 25 Nucleic Acids Res., 12:857-872 (1984). Plasmid HIMP, containing the cloned human DNA sequence as an EcoRI insert ligated into the EcoRI site of a Bluescript KS+ vector (Stratagene, LaJolla, CA) and transformed into E. coli DH-1 cells was deposited on July 29, 1988 with the 30 American Type Culture Collection, Rockville, Maryland under A.T.C.C. accession No. 67753.

35 As illustrated in Figure 2, the organization of the Chinese hamster cDNA is similar to that of the human clone and specifies a protein identical in size to the human protein, and contains the poly(A) and ATG consensus sequences.

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The mouse DNA fragment was sequenced from the 5'- end of both strands, and these sequences are set forth in Figures 3A and 3B, respectively. These sequences comprise 737 of the 750 nucleotide base pairs 5 comprising the mouse cDNA. The mouse cDNA sequence(s) display a high degree of similarity to the human cDNA sequence and correspond(s) to the region spanned by nucleotides 405-1157 of the human HIMP clone.

Figure 4 provides a comparison of the deduced 10 amino acid sequences of the human and Chinese hamster proteins and indicates a high level of conservation of the amino acid sequence information. The non-matching amino acids are surrounded by a box with those having similar chemical properties denoted by an asterisk. Of 15 the 514 amino acids specified by the two open reading frames, only eight amino acid differences are noted between the human and the Chinese hamster proteins. Furthermore, five out of eight of these amino acid changes are conservative with respect to the chemical 20 nature of the amino acid. This similarity in amino acid sequence is mirrored in the DNA sequences that show an 89% identity.

Similar results are also obtained from a comparison of the sequence information derived from the 25 mouse IMPDH cDNA, set forth in Figure 3A and 3B, and that derived from the human cDNA, set forth in Figure 1, wherein a 89% identity was observed. These results confirm the identity of the human, Chinese hamster, and mouse clones as those for IMPDH and demonstrate the high 30 conservation of the amino acid sequence of the enzyme among these three species. A comparison was made between the deduced amino acid sequence of the cloned human IMPDH cDNA with all sequences in the NBRF database by using the software provided by the 35 University of Wisconsin Genetics Computer Group [Devereux, et al., Nuc. Acids. Res., 12:387-395

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(1984)]. A degree of homology (score 347, 57% similarity over a stretch of 506 amino acids) was observed for the bacterial IMPDH protein. Three regions of 50 amino acids in the interior of the protein 5 sequence show a 56-67% identity in amino acid sequence. If allowance is made for amino acids of similar chemical nature, the similarity of these three regions is approximately 78%.

10 The similarity of the human, mouse, and Chinese hamster proteins indicates a functional selection for conservation of amino acid sequence. The dissimilarities between eukaryotic and prokaryotic amino acid sequences are indicative of a substantial lack of homology of DNA sequence.

15

#### EXAMPLE 5

20 Protease Digestion and Amino Terminal Sequencing of Purified Chinese Hamster IMPDH Protein  
25 Initial attempts to sequence the purified Chinese hamster IMPDH protein indicated that the amino terminus was blocked. In an attempt to further verify the identity of clones putatively coding for the IMPDH protein obtained in Example 4, a portion of the Chinese hamster protein purified according to Example 2 was subjected to protease digestion and amino acid sequencing as follows.

30 A sample of the purified Chinese hamster protein in 20 mM Tris-HCl, pH 8.0, 200 mM KCl, 10 mM DTT, and 0.25% SDS was heated at 65°C for 2 minutes. Staphylococcus aureus V-8 protease was added at a weight/weight ratio of protease to substrate of 1:100, and the solution was incubated at 37°C for two hours. The sample was then chilled to 0°C and centrifuged at 12,000 x g for two minutes. The resulting peptides in the supernatant were purified by reversed-phase high-

- 20 -

pressure liquid chromatography on a Synchropack C4 column (4.1 x 100 mm). Small aliquots (50  $\mu$ L) containing approximately 350 pmoles of digested enzyme were loaded to minimize the effects of SDS. The 5 peptides were eluted with a linear gradient (0-100%) of 60/39/1:acetonitrile/water/trifluoroacetic acid. Flow rate was 1 mL/min with a gradient duration of 30 minutes. Peptide peaks were collected in microfuge tubes, lyophilized, and stored at -70°C. Amino terminal 10 sequencing was performed at the Chicago Medical School, North Chicago, IL, by using an Applied Biosystems (Foster City, CA) protein sequencer, amino acid analyzer, and the accompanying protocols.

15 A sequence of 35 amino acids obtained by analysis of one of the peptides was compared with the protein sequence deduced from the human and Chinese hamster cDNA clones. This 35 amino acid segment, indicated by a box in Figure 1, corresponds to deduced amino acid residues 336-370 in both the human and 20 Chinese hamster proteins.

#### EXAMPLE 6

25 The Preparation and Use of an IMPDH DNA Construct as a Selectable Marker:

This example describes the preparation and use of IMPDH DNA constructs, which permit identification of cells that have incorporated a selective fragment of foreign DNA into their genetic material. The successful 30 practice of the procedures is based on the requirement of IMPDH as a normal constituent of the cell for cell survival and the knowledge that inhibitors of IMPDH can be cytotoxic to cells at concentrations of 0.1 to 0.5  $\mu$ g/mL. Increased cellular levels of IMPDH confer 35 resistance to IMPDH inhibitors and negate the cytotoxic effects of these agents.

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DNA sequences that can be readily combined with the IMPDH DNA sequence include any DNA desired sequences that can be ligated into the plasmid construct and that will not compromise the ability of the IMPDH 5 cDNA product to specify resistance to MPA (mycophenolic acid) or to other IMPDH inhibitors. The constructs can be incorporated into cells by using standard DNA transfection technology (Davis, et al., Molecular Biology, 18-1:286-289; Davis, L.G., et al., eds. Science 10 Publishing Co., Inc.; Elsevier, N.Y. 1986). After transfection, the addition of an IMPDH inhibitor to the culture medium will permit the growth of only those cells that have incorporated the construct into their genetic material. Cells that have not acquired the 15 construct will be killed by the IMPDH inhibitor.

DNA sequences coding for the IMPDH enzyme were ligated into the pMSG plasmid (Pharmacia, Inc., Piscataway, NJ), which contains appropriate expression sequences and the DNA sequence coding for the E. coli 20 gpt protein. A SmaI-EcoRV fragment derived from the HIMP plasmid was subcloned into the SmaI site of the pMSG plasmid. This process placed the IMPDH cDNA under the control of a dexametasonone-inducible mouse mammary tumor virus promoter. This plasmid construct was then 25 introduced into V15 hamster cells (derived by mutagen treatment of Chinese hamster V79 cells and having no detectable HGPRT activity) by using the calcium phosphate DNA transfection technique described in Davis, et al., Molecular Biology, 18-1:286-289 (1986), supra.

30 After introduction of the construct into the hamster cells, mycophenolic acid (2  $\mu$ g/mL) was added to the culture medium to select for those cells that had integrated the construct into their genetic material. Those cells that have integrated the construct into 35 their genetic material produce the IMPDH enzyme and are therefore resistant to the cytotoxic effects of

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mycophenolic acid. A cell clone designated IMP1 was isolated; this clone was resistant to mycophenolic acid, indicating that the cells had incorporated the construct and were over-producing the IMPDH enzyme.

5 To verify that the resistance resulted from the incorporation of the construct, the MPA-resistant cells were successfully transferred to HAT medium. [Dulbecco's modified MEM containing 5% fetal bovine serum, aminopterin (2  $\mu$ g/mL), and mycophenolic acid (25  $\mu$ g/mL), and supplemented with hypoxanthine (15  $\mu$ g/mL), thymidine (10  $\mu$ g/mL), and xanthine (250  $\mu$ g/mL)]. The ability of the cells to grow in the selective medium is attributable to the production of the E. coli gpt enzyme, which catalyzes the production of xanthine

10 monophosphate from the reaction of xanthine and phosphoribosyl pyrophosphate. Presence of the enzyme compensates for the purine de novo synthetic block imposed by the presence of aminopterin and MPA. The dual resistance to mycophenolic acid and HAT selection

15 displayed by the transformed host is evidence that the resistance is attributable to the incorporation of DNA of the plasmid construct. Growth of the cells for several generations in the absence of mycophenolic acid did not decrease the resistance of the cell clone to

20 MPA, indicating that the construct DNA was incorporated into host chromosomal material.

25

The IMPDH expression in the IMP1 cell clone and the V15 parent was examined to further define the basis for the mycophenolic acid resistance. A five-fold increase in the IMPDH activity in cell homogenates was observed in the IMP1 cells relative to the V15 parent. The cellular protein in these cell homogenates was electrophoresed through polyacrylamide gels, transferred to nitrocellulose and the amount of IMPDH enzyme was quantitated by immunoblot analysis with the anti-IMPDH antiserum prepared according to Example 3. In both the

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V15 and IMPl cells, the antiserum reacted with a protein of 56 kDa corresponding to IMPDH. The amount of IMPDH enzyme was approximately five-fold higher in the IMPl cells than in the V15 parent. Furthermore, the relative 5 gel migration distance of the two proteins was identical, suggesting the IMPDH protein produced in the IMPl cells has the same molecular weight as the V15 enzyme.

The amount of IMPDH mRNA in the parent and 10 transformed cell lines was quantitated by Northern blot analysis by using a human IMPDH cDNA probe, prepared according to Example 4. Total cellular RNA was isolated by disrupting cells in guanidinium lysis buffer, pH 7.0, composed of 4 M guanidinium thiocyanate, 50 mM potassium 15 acetate, 0.1 M 8-mercaptopethanol, and 0.5% sarcosyl. The RNA was purified by centrifugation through a CsCl cushion as described by Chrigwin, *et al.*, Biochemistry, 18:5294-5299 (1979). Hybridization signals corresponding to a 2.2 kilobase message were detected in 20 both the IMPl and V15 cells. However, the IMPl cells contained an additional hybridization band corresponding to a message size of approximately 2.0 kilobases. This is the approximate message size expected for transcription of IMPDH mRNA from the plasmid construct. 25 These results show that the IMPl cells overproduce an IMPDH enzyme that is indistinguishable, by polyacrylamide gel electrophoresis, from that produced by V15 cells, and suggest that the increased IMPDH is a result of transcription from the plasmid construct containing 30 the human IMPDH cDNA.

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The foregoing example demonstrates that the selectable marker system of the invention provides a convenient means to study and obtain a regulated expression of virtually any selected foreign DNA.

5 Moreover IMPDH is a dominant marker and no requirement for use of deficient hosts exists.

EXAMPLE 7

10 Analysis of IMPDH Expression in Normal and Malignant Cells

To determine whether increased amounts of IMPDH mRNA are the cause of the elevated levels of IMPDH in tumor cells, total cellular RNA from a variety of growing human leukemic cell lines and in normal peripheral blood granulocytes and lymphocytes was examined by Northern blot analysis through the use of the human cDNA as described in Example 6.

Cells were grown in Dulbecco's modified Eagle's medium or RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 µg/mL), fungizone (0.25 µg/mL), and glutamine (2 mM) in a humidified incubator supplied with a constant amount of 8% CO<sub>2</sub> in air. Peripheral blood leukocytes were isolated from freshly drawn peripheral blood by Histoplaque-1077 (Sigma Chemical Co., St. Louis, MO) gradient centrifugation as described in Boyum, A., Scand. J. Clin. Lab. Invest., 21(97):51-55 (1968); and Klock, J.C., and Bainton, D.F., Blood, 48:149-161 (1976). The mononuclear fraction contained predominately lymphocytes (85-95%), with monocytes comprising the remaining percentage. Immediately after purification, the lymphocytes were resuspended in appropriate buffers for either Western blot analysis or for isolation of total cellular RNA.

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A reduced level of IMPDH expression was consistently observed for the RNA isolated from lymphocytes relative to the leukemic cell lines that had markedly increased expression of a 2.2 kb transcript 5 corresponding to the IMPDH message. A similar pattern was observed for the amounts of cellular IMPDH detected with the specific IMPDH antibody in conjunction with the Western blotting technique. These results were similar in that much higher amounts of the IMPDH protein were 10 observed in the leukemic cells relative to the peripheral blood cells. Similar results were also obtained for measurements of the enzyme activity in these cell lines. These marked differences in the expression, amount, and activity, between the normal and 15 leukemic cells may be associated with the absence of cell replication in the normal cells and the active proliferation of the leukemic cells.

Cultured normal human fibroblast and sarcoma cells were similarly analyzed. The differences in IMPDH expression between the normal and tumor cells were not as great as those observed between the leukemic cells and the normal peripheral blood cells. However, all of the sarcoma cells had higher levels of mRNA expression, larger amounts of the protein, and greater IMPDH 25 activity than the normal fibroblasts. These differences may again be attributable in part to a difference in the growth rate of the various cell types because the 37-h doubling time of the normal fibroblasts is greater than that observed for the sarcoma cells. However, other 30 factors appear to influence the IMPDH expression because an absolute correlation between IMPDH activity and cellular growth rate was not always observed for the tumor cell lines.

The foregoing illustrative examples relate, in part, to the isolation of cDNA sequences encoding mouse, Chinese hamster, and human species IMPDH proteins.

Those skilled in the art will readily appreciate that

5 the DNA and deduced amino acid sequence information provided herein make available numerous other forms of DNA sequences, such as genomic sequences obtainable by hybridization screening of genomic libraries through the use of DNA probes designed by using the sequence  
10 information of Figures 1-3, or manufactured DNA sequences synthesized from nucleic acids and potentially including alternate (degenerate) codons specifying the same amino acids, or DNA sequences comprising, e.g., part cDNA and part manufactured DNA. In a like manner,  
15 the DNA sequence information provided herein enables the isolation of other eukaryotic DNAs encoding IMPDH such as avian (chicken, turkey), fish, and mammalian (bovine, ovine, porcine) species DNAs by means of hybridization screening under appropriate stringency conditions.

20 The availability of the above-noted DNA sequences allows for preparation of IMPDH by in vitro transcription and translation of the DNA and for the development of a wide variety of viral or circular plasmid DNA vectors useful both for the biological  
25 amplification of the DNA and for the securing of recombinant expression of the DNA of proteins having IMPDH biological activities in prokaryotic and, especially, eukaryotic, host cells and organisms.

30 Well-known recombinant means for introducing genes into host cells and organisms have been described. For example, Palmiter, et al., Science, 222:809 (1983), have described transgenic mice containing the human growth hormone gene fused to a promoter sequence. MacLean, N., et al., Bio/Tech., 5:257 (1987), have produced transgenic fish. Caplan, A., et al., Science, 222:815-821 (1983), described the  
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use of a modified plasmid for use as a vector to transfer foreign genes into plants. More recently, Sinkar, V.P., et al., Genes & Development, 2:688-697 (1988), described transgenic tobacco plants. These recombinant means are expected to allow for the production of plant or animal organisms into which IMPDH encoding DNA has been introduced and which therefore contain increased endogenous levels of guanosine-5'-monophosphate (GMP). GMP, a natural constituent of all living materials and normally present only in trace amounts, is a member of a family of flavor potentiators commonly used as food additives. GMP enhances the taste intensity of certain flavors and can suppress the perception of a sour or bitter taste. [Heath, et al., "Flavor Chemistry and Technology", AVI Publishing Co., Westport, CT., (1986)]. When combined with the commonly used food additive, monosodium glutamate, GMP acts synergistically to enhance flavor and it is therefore possible to enhance the taste properties of certain foods by increasing the endogenous GMP levels. Organisms with increased levels of GMP can also provide a ready source for the isolation and extraction of GMP for use as a food additive. Studies of tissue culture cells with altered levels of IMPDH activity show an association between increased IMPDH activity and elevated GMP levels. [Ullman, J. Biol. Chem., 258:523-528 (1983)]. Thus, selection for an organism with increased levels of IMPDH activity simultaneously selects for organisms with elevated tissue levels of GMP.

Microinjection and other transformation techniques are expected to readily allow for incorporation of extra copies of IMPDH encoding DNA into host cells and organisms, with exposure of the cells and organisms to inhibitors such as MPA providing a basis for selection of those cells having incorporated the desired sequences.

Cells and organisms having enhanced IMPDH production levels are, of course, also made available according to the present invention by means of the screening and selection procedures applied in Example

- 5 1. Briefly, a selected somatic or embryonic cell type is (either with or without prior exposure to mutagenic influences) screened in culture for the capacity to survive in the presence of elevated levels of an inhibitor such as MPA. Cells capable of surviving in
- 10 the screening environment, e.g., at levels of MPA of 0.5 to 1.0  $\mu$ L/mL, are thereafter subjected to stepwise incremental selection at much higher levels of the inhibitor. The resulting cells and organisms having enhanced IMPDH synthetic capacity vis-a-vis parent cells
- 15 will also be expected to display enhanced capacity for synthesis of GMP.

Among the additional forms of DNA provided by the invention are those that encode allelic variants of the specific mammalian IMPDH protein of Figures 1 and 2, as well as analog proteins that possess one or more variations in IMPDH biological activities.

Protein and peptide products of the invention include not only those produced as recombinant expression products of "full-length" and fragmentary DNA sequences of the invention but also those that are prepared by chemical synthesis from amino acids. As one example, analysis of the deduced amino acid sequences of IMPDH proteins is expected to provide valuable information concerning potential antigenic epitopes present therein, allowing for the preparation of synthetic antigenic peptides duplicative of about 6 to 20 continuous residues of the protein. These, in turn, are expected to allow for the preparation of monospecific polyclonal and monoclonal antibodies useful in the quantitative detection of IMPDH proteins. Further, it is anticipated that the deduced amino acid

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sequenc s information can be used to modify existing drugs and to design new drugs as inhibitors of IMPDH activity.

5 Numerous modifications and variations in the invention as described in the above illustrative examples are expected to occur to those skilled in the art; consequently, only such limitations as appear in the appended claims should be placed thereon.

10 Accordingly, it is intended in the appended claims to cover all such equivalent variations that come within the scope of the invention as claimed.

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- 30 -

WHAT IS CLAIMED IS:

5 1. A purified and isolated DNA sequence  
encoding eukaryotic IMPDH.

10 2. The DNA sequence according to claim 1  
encoding human IMPDH.

15 3. The DNA sequence according to claim 1  
encoding mouse IMPDH.

15 4. The DNA sequence according to claim 1  
encoding Chinese hamster IMPDH.

20 5. The DNA sequence according to claim 1  
which is a cDNA sequence.

25 6. The DNA sequence according to claim 1  
which is a genomic DNA sequence.

7. The DNA sequence according to claim 1  
which is a manufactured DNA sequence.

25 8. The DNA sequence according to claim 1  
selected from the group consisting of the DNA sequences  
set out in Figures 1 and 2.

30 9. A prokaryotic or eukaryotic host cell  
transformed or transfected with a DNA sequence according  
to claim 1.

35 10. The prokaryotic or eukaryotic host cell  
according to claim 9 wherein said host cell is a Chinese  
hamster cell.

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11. The prokaryotic or eukaryotic host cell according to claim 9 wherein said host is an E. coli DH-1 cell having A.T.C.C. accession No. 67753.

5 12. The host cell according to claim 9 having an enhanced capacity for production of IMPDH.

13. The host cell according to claim 9 having enhanced capacity for production of GMP.

10

14. A viral or circular DNA plasmid vector comprising an IMPDH encoding DNA sequence according to claim 1.

15 15. The vector according to claim 14 further comprising an expression control DNA sequence operatively associated with said IMPDH encoding DNA.

20 16. A plant or animal comprising cells transfected or transformed with a DNA sequence according to claim 1.

25 17. A method for the production of IMPDH comprising:  
growing, under suitable culture conditions, a host cell transformed or transfected with a DNA sequence according to claim 1; and  
isolating from said host cell or its culture medium the polypeptide product of the expression of said DNA sequence in said host.

30 18. A method for the production of IMPDH comprising:

35 disposing a DNA sequence according to claim 1 in a cell-free transcription and translation system; and

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isolating from said system the polyp ptide product of the expression of said DNA sequence.

19. A method for the production of GMP  
5 comprising:

growing, under suitable culture conditions, a host cell transformed or transfected with a DNA sequence according to claim 1 in a manner allowing expression of IMPDH in said host; and

10 isolating from said host cell or its culture medium GMP produced by said.

20. A method for generating eukaryotic cell variants with altered IMPDH activity comprising the  
15 steps of:

(1) selecting colonies of viable cells in the presence of cytotoxic quantities of an IMPDH inhibitor; and

20 (2) further subjecting colonies selected according to step (1) to stepwise incremental selection in the presence of elevated cytotoxic quantities of an IMPDH inhibitor.

25 21. The method of claim 20 including subjecting said cells to mutagenic influences prior to step (1).

30 22. The method of claim 21 wherein said cells are Chinese hamster cells, said mutagen influence is NMNG and said cytotoxic inhibitor is a member selected from the group consisting of mycophenolic acid, ribavirin, brenidin, and tiazofurin.

35 23. The peptide product of the in vitro or in vivo expression of a DNA sequence according to claim 1.

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24. A synthetic peptide duplicative of a sequence of amino acids present in and providing at least one antigenic epitope of eukaryotic IMPDH.

5 25. An antibody immunoreactive with the peptides of claim 24 and immunoreactive with IMPDH.

10 26. A monoclonal antibody according to claim 25.

15 27. A polyclonal antibody according to claim 25.

28. A method for the quantitative detection 15 of IMPDH encoding DNA or RNA and based on hybridization of said nucleic acids with a DNA sequence according to claim 1.

20 29. A method for incorporating a selected fragment of foreign DNA into the genetic material of a cell, which comprises:

25 forming a recombinant construct by fusing a DNA sequence according to claim 1 to said selected fragment of foreign DNA to be incorporated,

transforming or transfecting said cell with said construct,

growing said transformed or transfected cell in a culture medium comprising a cytotoxic IMPDH inhibitor, and

30 selecting from among said transformed or transfected cells those cells which are resistant to said cytotoxic IMPDH inhibitor.

35 30. The method according to claim 29 wherein said cytotoxic IMPDH inhibitor is a member selected from the group consisting of mycophenolic acid, ribavirin, brnidin, and tiazofurin.

1   GGGGGGTCTGGAGAACGGCCCTACGTCCTGTCCTGTTGCCATGGCCGACTACCTGATT   60  
 61   GAGGGGACCTCCTAACGGCCAGACGGACTCACACCAACAGGACTCTCAACTGCC   120  
 121   GAGACGGCCTCACCTAACATGAACTTCTCATTCAGCTGGTACATCGACCTTCACGGAG   180  
 181   QVDPALTSTGACTCTGCTCTGACCCAGGAAATCACTCTTAAGACCCACTGGTTT   240  
 241   CCTCTCCATGGACACAGTCACAGGGCTGGCATGGCAATAGCAATGGCCCTACAGGCC   300  
 301   CTATGGCTCATCCACCAACTGTACACCTGAAATTCCAGGCCATGAAAGTTGCCAAG   360  
 I   G   F   I   H   H   N   C   T   P   E   F   Q   A   N   E   V   R   K   V

FIGURE 1A

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361	TGAAAGAAATATGAAACAGGGATTCAACAGACCCCTGTGGGCTCAGCCCCAGGATCGCG	420
	K R Y E Q G F I T D P V V L S P R D R V	
421	TGCCGGATGTTTGTAGGCCAAGCCCCGGCATGGTTCTGCCGTATCCCAATCACAGACA	480
	R D V F E A K A R H G F C G I P I T D T	
481	CAGGCCGGATGGGAGGCCATCTCTCCAGGGACATTGATTCTCTCA	540
	G R H G S R L V G I I S S R D I D F L K	
541	AAACAGGAGAACATGACTGTTCTTGAGAGATAATGACAAAGAGCCAAAGACTTCTCG	600
	E E H D C F L E E I M T K R E D L V V	
601	TAGCCCCCGCAGCATCACACTGAAAGGCCAAATGAAATTCCTCTGCAGGCCAGGAAGC	660
	A P R S I T L K E A N E I L Q R S K K G	
661	GAAGAGTGCCTATTGTAATGAAAGATGATGAGCTTGTCGCCATCATTCGGGACAGACC	720
	K L P I V N E D D E L V A I I A R T D L	
721	TGAAGAAATCGGGACTTACCTAGCCCTCCAAAGATGCCAAGAAACAGCTGCTGTGTC	780
	K R N R D Y P L A S K D A K Q L L C G	

FIGURE 1B

SUBSTITUTE SHEET

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781	GGGCAAGCCATGGCACTCATGAGGATGACAAGTATAGGCTGGACTTGGCTGGCCAGGCTG	840
	A A I G T H E D D K Y R L D L L A Q A G	
841	CTGTGATGTAGTGGTTGGACTCTTCCAGGAAATTCCATCTTCCAGATCAATATCA	
	V D V V L D S S Q G N S I F Q I N H I	900
901	TCAAGTACATCAGAACAAATACCCATACTCCAAAGTCATTGGAGGCCAATGTGGTCACTG	
	K Y I K D R V P N L Q V I G G N V V T A	960
961	CTGCCCAAGGCCAAGAACCTCATGTGATGAGGTGTCGGATGCCCTGGGGCATGGAA	
	A Q A K N L I D A G V D A L R V G H G S	1020
1021	GTGGCTCCATCTGCATTAACCCAGGAAGTGCTGGCCCTGGGGCCCCCAAGCAACAGAG	
	C S I C I T Q E <u>V</u> L A C C R P Q A T A V	1080
1081	TGTAACTAGGTGTCAAGCTAGCTATGGCAAGGGCCCTTGGCTGGTCAATTGGCTGATGGGAA	
	Y K V S E Y A R F G V P V I A D C C I	1140

FIGURE 1C

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1141 TCCAAATGTTGGTCATTGCCAAACCCRTGGCCCTTGGGCTCCACAGTCATGG  
0 N V G H I A K A L G A S T V M M G 1200

1201 GCTCTCTGGCTGCCACACTGAGCCCCCTGGTGAATACTTCTTCCGATGGATCC  
 S L L A A T T E A P G E Y F P S D G I R 1260

1261 GGCTAAAGAAATATCGGGTATGGGTTCTCGATGCCATGGACAAAGCACCTCAGGCC  
 L K R Y R C H G S L D A M D K H L S S Q 1320

1321 AGAAACGATATTCAGTGAAGCTGACAAATCAAAGTGGCCCAAGGGAGTGTCTGGCTG  
 N R Y F S E A D K I K V A Q G V S G A V 1380

1381 TGCAGGACAAAGGTCATCCACAAATTGGTCCCTGATTGCTGGCATCCAACT  
 Q D K G S I H K F V P Y L I A G I Q H S 1440

1441 CATGCCAGGACATTGGTGCCAAAGAGCTTGAACCATGTTACTCTGGGG  
 C Q D I G A K S L T Q V R A M Y S C E 1500

FIGURE 1D

SUBSTITUTE SHEET

1501 AGCTTAAGTTGACAAGAGAACGTCCTCAGCCCAGGTGGAAAGGTGGCTCCATAGCCCTCC  
L R F E K R T S S A Q V E G G V H S L H 1560

1561 ATTCGGTATGAGAAAGGGCTTTCTGAAAAGGGATCCAGCACACCTCCTCGGTTTTTTT 1620  
S Y E K R L F

1621 CAAATAAAAGTTAGAAAGACCC 1642

FIGURE 1E

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Chinese hamster IMP dehydrogenase cDNA sequence and  
translation, clone CIMP.

1	CACGGGTCCGTCTCGTGGCCATGGGGACTACCTGATTAGGGAGGACATCTTA	60
	M A D Y L I S G G T S Y	
61	CGTGGCCGACCGACGGGCTCACAGGGCAGCAGCTCTCAACTGGGGATGGCCTCACCTA	120
	V P D G L T A Q Q L F N C G D G L T Y	
121	CAACGATTTCATTCTCATTCTGGTATATCGACTTCACACTGCCGACCAAGTGGATTGAC	180
	N D F L I L P G Y I D F T A D Q V D L T	
181	CTCTGCTCTAAAGAAGATCACCCACTGAAGACCCACTGGTTCCACCTATGGACAC	240
	S A L T K I T L K T P L V S S P M D T	
241	TGTCACAGGGCTGGAATGGCCATTGCAATGGCTTACAGGAGGTATTGGCTTCATCCA	300
	V T E A G M A I A M A L T G G I G F I H	
301	CCACAACTGTACACCTGAATTCCAGGCCAATGAAGTTCGGAAGTAAAGAAATATGAACA	360
	H N C T P E F Q A N E V R K V K Y E Q	

FIGURE 2A

361 GGGATTCAACTGATCCTGAGTCCTTAGCCCCAAGGATCGTGTGAGGGATGTTTGA 420  
 G F I T D P V L S P K D R V R D V F E

421 AGCCAAAGCCAGGCATGGCTTCTGTGGTATCCCCATCACAGATAACAGGCCGGATGGGAG 480  
 A K A R H G F C G I P I T D T G R M G S

481 TCGACTGGGGCATTCATTCTCAAGGGATATTGATTCTCAAGGAGGAAGGAGCATGTA 540  
 R L V G I I S S R D I D F L K E E E H D

541 CCGTTTCTGGAGGAGATCATGACAAAGAGGGAAAGGATTGGTGGCTGGCCCTGCAGGCAT 600  
 R F L E E I M T K R E D L V V A P A G I

601 CACTCTGAAGGAGGCAAATGAAATTCTGCAGGCCAGTAAAGGAAAGTGTGCCCATGT 660  
 T L K E A N E I L Q R S K K G K L P I V

661 GAATGAAAAATGATGAGCTGGTAGCCATTCGCTGGACAGACCTGAAGGAAGAATCGTGA 720  
 N E N D E L V A I I A R T D L K K N R D

721 TTACCCATTGGCTTCCAAAGATGCCAAAGCAGCTACTATGTGGGGCAGCCATTGGTAC 780  
 Y P L A S K D A K K Q L L C G A A I G T

FIGURE 2B

781 TCATGAGGATGACAAGTATAGGCTGGACTTACTGGCTCTTGGATGTGGTGGT 840  
 H E D D K Y R L D L A L A G V D V V V

841 TTTGGACTCTCCCAGGGAAACTCCATTTCATTCAAAATGATCAATACATGAAAGA 900  
 L D S S Q G N S I F Q I N M I K Y M K E

901 GAAATACCCCCAATCTCCAAGTCATGGAGGCCAATGTAGTCACTGCTCAAGCCAAGAA 960  
 K Y P N L Q V I G G N V V T A A Q A K N

961 CCTCATAGACGCCAGGTGTTGGATGCTCTGGCAGTTGGCATGGGGTGTGGTTCCATCTGCAT 1020  
 L I D A G V D A L R V G M G C G S I C I

1021 TACTCAGGAAGTGTGGCTGTGGTGGCCCAAGCAACAGCAGTGTACAAGGGTTCTGTA 1080  
 T Q E V L A C G R P Q A T A V Y K V S E

1081 GTATGCTCGGGCTTTGGTGTCCCTGTATTGCTGATGGAGGAATCCAAAATGTGGGTCA 1140  
 Y A R R F G V P V I A D G G I Q N V G H

1141 TATTGCCAAAGCTTTGGAGCTCTACAGTCATGATGGGCTCCCTTGGCTGC 1200  
 I A K A L A S T V M M G S L L A A

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FIGURE 2C

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1201	CACCACCGAAGCCCCCTGGTGAGTACTTCTCAGATGGGATCCGGCTAAAAAAGTACCG	1260
	T T E A P G E Y F F S D G I R L K K Y R	
1261	TGGTATGGGTCTTGATGCCATGGACAAGCATCTCAGGCCAGAACCGATATITCAG	1320
	G M S L D A M D K H L S S Q N R Y F S	
1321	TGAAGCTGACAAATCAAAGTGGCCCAAGGAGTTCAAGGAGCTGTGCAGGACAAAGGGTC	1380
	E A D K I K V A Q G V S G A V Q D K G S	
1381	TATCCACAAAGTTCGTCCCTTATTGATTGCTGGCATCCAGCATTCCTGTCAGGACATGG	1440
	I H K F V P Y L I A G I Q H S C Q D I G	
1441	TGCCAAGAGTTAACCCAAAGTCAGAGCCATGATGTTACTCTGGGAACCTCAAGTTGAGAA	1500
	A K S L T Q V R A M M Y S G E L K F E K	
1501	GAGAACATCCTCAGCTCAGGTGGAAAGGTGGTCCACAGCCTTCAATTGTATGAGAAGCG	1560
	R T S S A Q V E G G V H S L H S Y E K R	
1561	GCTTTCTGAAAGAGATCCAGTATATGCCCTGAAATTTCATAAAAGTTTGAAAAAA	1620
	L F	

FIGURE 2D

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## MOUSE IMP DEHYDROGENASE, SEQUENCE M1.

1 CGCCCCAAGGATCGTGTACGGCGATGTTTGAGGCCAAGGCCAGGGATGGCTCTGTGCT  
 60 GGGGGTCCCTAGCACATGGCTACAAAACCTCCGGTTACCGTAGCGTAAGGACACCA

61 ATCCCCATCACAGATAAGGCCGGATGGGAGTCGATTGGTGGGCATCATCTCCTCAAGG  
 TAGGGGTAGTGTCTATGTCGGCCTACCCCTCAGCTAACCAACCCGTAGTAGGGAGTTCC

121 GACATTGATTCCCTCAAGGAGGAAGAGGCATGACCGGGTCTTGGAAAGAGATCATGACTAAG  
 CTGTAACTAAGGAGTTCCCTCGTACTGGCCAAGAACCTCTCTAGTACTGATTC

181 AGGAAAGATTGGTGGCCCTGCCGGTCACTCTGAAAGAGGCAAATGAGGATTCTG  
 TCCCTCTAACCAACCAGGGGACGGCCAGTGGACTTCTCCGTTACTCTAAGAC

241 CAGCGAAGTAAAGGAAAGTTGCCATTGTGAATGAAATGATGAGGCTGGTAGCCATC  
 GTCGCTTCATTTCCCTTCAACGGGTAAACACTTACTTTACTACTCGACCATCGGTAG

301 ATTGCCCGGACAGACCTAAAGAATCGTGAATTACCCCCCTGGC  
 TAAACGGGCTGTCTGGATTCTTAGCACTAAATGGGGACCG

FIGURE 3A

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## MOUSE IMP DEHYDROGENASE, SEQUENCE M12.

1	CGGACCCACATTGGATTCCTCCATCAGCAATAACAGGAACACCAAAAGCGACGGGCATA		
	GCCTGGGTGAAACCTAAGGAGGTAGTCGTTATTGTCCTGGTTTCGCTGCCGGTAT		60
61	CTCAGAGACCTTGTACACTGCTGGCTTGGGGCCACAGGGCCAAACACTTCTGGGT		
	GAGTCTCTGGAACATGTGACGACACCAGAACCCGGGGTCCGGGTGTGAAGGACCCA		
121	GATGCAGATGGAACCACTTCCCATGCCGACTCGCAAAGCATCTACACCTGCATCTATGAG		
	CTACGGTCTACCTTGGTGAAGGGTACGGCTTGAGCGTTCTGTAGATGTGGACGTAGATACTC		120
181	GTTCTTGGCTTGGCAGGAGGTGACTACATTGCCTCCATGACCTGTAAGCTGGATACT		
	CAAGAACCGAACGGCGTCCACTGTGTAACGGAGGTACTGGACATCTGACCCCTATGAG		180
241	TCTCCTGTATTTGATCATATTGAGTTGGAAAGATGGAGTTCCCTGGGAAGAGTCCTA		
	AGAGGAACATACATAACTAAACCTTACACTACCTAAAGGGACCCCTCTCAGGTA		240
			300

FIGURE 3B

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301 AAACCACTACATCCACACCAGCAAGGGCCAGTAAAGTCAGGCCATACTTGTCA~~T~~CAT  
360 TTTGGTGTAGGTGTGGTCTCCGGTCATTCAAGTCGGATATGAACAGTAGGAAGTA

361 GAGTGCCAATGGCTGCCACACAGCAGTGTGCTT  
393 CTCACGGTTACCGACGGGTGTGTCAACGAA

FIGURE 3B (CON'T)

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human	1	MADYLISGGTSYVPDDGLTAQQLFNCGGDGLTYNDFLILPGYIDDFTADQVDLTSALTKKIT	
hamster	1	MADYLISGGTSYVPDDGLTAQQLFNCGGDGLTYNDFLILPGYIDDFTADQVDLTSALTKKIT	60
	61	LKTPPLVSSSPMDTVEAGMAIAMALTGGIGF <small>I</small> AMALTGGIGF <small>I</small> HNNCTPEFQANEVRKVKKYEQGFITDPVV	
	121	LSPKDRVRDVFEAKARHGF <small>C</small> FGIPITDTGRMGSRLVGISSR <small>D</small> IDFLKEEEHD <small>C</small> FLSEEIMT	120
	181	KREDLVVAPRSITLKEANEILQRSKKGKLP <small>I</small> VNEEDPELVALIARTDLKKNRD <small>D</small> YPLASKDA	
	241	KKQLLCCGAAIGTHEDDKYRDLLAQAGVDDVVVLDSQQGNSIFQINMIKKYIKDKYPNLQVI	
		KKQLLCCGAAIGTHEDDKYRDLLAQAGVDDVVVLDSQQGNSIFQINMIKKYIKDKYPNLQVI	300

FIGURE 4A

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301 GGNVVTAQAKNLIDAGVDALRVGMCSICITQEVLACGRPQATAVYKVSEYARRFGV  
GGNVVTAQAKNLIDAGVDALRVGMQCGSICITQEVLACGRPQATAVYKVSEYARRFGV 360

361 VIADGGIQN VGHIAKALALGASTVMMGSLLAATTEAPGEYFFSDGIRLK KYRGMGSLDAM  
VIADGGIQN VGHIAKALALGASTVMMGSLLAATTEAPGEYFFSDGIRLK KYRGMGSLDAM 420

421 DKHLSSQNRYFSEADKIKVAQGVSGAVQDKGSIHKFVPYLIAGIQHSCQDIGAKSLTQVR  
DKHLSSQNRYFSEADKIKVAQGVSGAVQDKGSIHKFVPYLIAGIQHSCQDIGAKSLTQVR 480

481 AMMYSGELKFEKRTSSAQVEGGVHSLHSYEKRLF  
AMMYSGELKFEKRTSSAQVEGGVHSLHSYEKRLF 514

FIGURE 4B

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>6</sup>  
 According to International Patent Classification (IPC) or to both National Classification and IPC  
 IPC(4) C12N 15/00 C12N 7/00 C12N 5/00 C07H 15/12  
 C12P 21/00

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>7</sup>

Classification System	Classification Symbols
435	68, 172.3, 235, 240.2 252.8, 252.1
536	27

Documentation Searched other than Minimum Documentation  
 to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>

Chemical Abstract data base (CAS) 1967-1989  
 Biosis database 1969-1989  
 Keywords IMPDH, sequence, clone

III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X, P	J. Biol. Chem., Vol. 263 issued 25 October 1988 (Baltimore, MD) Collart et al. Cloning and Sequence Analysis of the Human and Chinese Hamster Inosine-5'-monophosphate Dehydrogenase cDNA's see entire document	1-23, 29-30
Y	Proc. Natl. Acad. Sci., Vol 78 issued May 1981 (Washington, D.C.) Huberman et al Mutagen-induced resistance to mycophenolic acid in hamster cells can be associated with increased inosine 5'-phosphate dehydrogenase activity. see entire document.	1-30

\* Special categories of cited documents: <sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

17 October 1989

Date of Mailing of this International Search Report

21 NOV 1989

International Searching Authority

ISA/US

Signature of Authorized Officer

*Patricia Carson*  
 Patricia Carson

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Analytical Biochemistry Vol. 150 issued 1985 (Great Britain) Ikegami et al. Direct Assay Method for Inosine 5'-Monophosphate Dehydrogenase Activity (see entire document)	1-30
Y	Biochemistry Vol. 191 issued 1980 (Great Britain) Gilbert et al. Active site modification of native and mutant forms of inosine 5' monophosphate dehydrogenase from <u>Escherichia coli</u> k12 (see entire document)	1-23,29-30
A	Nucleic Acids Res. Vol. 6 issued 1985 (Oxford, England) Francoise et al. The expression in <u>E coli</u> of a synthetic gene encoding somatomedin-C (IGF-1) (see pages 1925-1926)	7,24
Y	USA 4,358,535 (Falkow et al) 9 Nov. 1982	28
Y	USA 4,404,279. (Giulia et al) 13 Sept. 1983	25-27